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High sensitivity mapping of methylated cytosines

Susan J.Clark[1,2,*], Janet Harrison[1], Cheryl L.Paul[1] and Marianne Frommer[3]
[1]Kanematsu Laboratories, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, [2]CSIRO Division of Biomolecular Engineering, Sydney Laboratory, PO Box 184, North Ryde, NSW 2113 and [3]School of Biological Sciences, A12, University of Sydney, NSW 2006, Australia

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ABSTRACT

An understanding of DNA methylation and its potential role in gene control during development, aging and cancer has been hampered by a lack of sensitive methods which can resolve exact methylation patterns from only small quantities of DNA. We have now developed a genomic sequencing technique which is capable of detecting every methylated cytosine on both strands of any target sequence, using DNA isolated from fewer than 100 cells. In this method, sodium bisulphite is used to convert cytosine residues to uracil residues in single-stranded DNA, under conditions whereby 5-methylcytosine remains non-reactive. The converted DNA is amplified with specific primers and sequenced. All the cytosine residues remaining in the sequence represent previously methylated cytosines in the genome. The work described has defined procedures that maximise the efficiency of denaturation, bisulphite conversion and amplification, to permit methylation mapping of single genes from small amounts of genomic DNA, readily available from germ cells and early developmental stages.

INTRODUCTION

Although 5-methylcytosine (5-MeC) was the first modified base to be discovered, nearly fifty years ago (1), its precise function and significance in the control of gene expression of higher organisms has remained elusive. The lack of information on the role of 5-MeC has been due, in part, to a lack of suitable detection techniques. Recently, however, there have been major improvements in the methods available for determining the methylation status of single cytosine residues in genomic DNA (2,3). Most of the methods developed to date depend upon cleavage of the phosphodiester bond alongside cytosine residues, using either methylation-sensitive restriction enzymes or reactive chemicals such as hydrazine which differentiate between cytosine and its 5-methyl derivative. The use of methylation-sensitive enzymes (4,5) suffers from the disadvantage that it is not of general applicability, since only a limited proportion of potentially methylated sites in the genome can be analysed and, in general, hemi-methylation remains undetected. Current genomic sequencing protocols (6,7), which identify a 5-MeC residue in genomic DNA as a site that is not cleaved by any of the Maxam and Gilbert sequencing reactions (8), are a substantial improvement on the original genomic sequencing method (9), but still have a number of disadvantages, including the requirement for a relatively large amount of genomic DNA and the necessity to detect a gap in a sequencing ladder which may contain bands of varying intensity and shadow bands.

A different approach, developed recently in our laboratory (10), relies on the ability of sodium bisulphite to efficiently convert cytosine residues to uracil in single-stranded DNA, under conditions whereby 5-MeC remains essentially non-reactive (11,12). The DNA sequence under investigation is then amplified by PCR with two sets of strand-specific primers to yield a pair of fragments, one derived from each strand, in which all uracil and thymine residues have been amplified as thymine and only 5-MeC residues have been amplified as cytosine. This method for identifying 5-MeC is therefore a positive one, in which the position of each 5-MeC residue is given by a distinct band on a sequencing gel.

Cytosine forms adducts across the 5–6 bond with a number of oxidation reagents including bisulphite ion (13). The deamination of cytosine by sodium bisulphite (Fig. 1) involves the following steps: (1) addition of bisulphite to the 5–6 double bond of cytosine, (2) hydrolytic deamination of the resulting cytosine-bisulphite derivative to give a uracil-bisulphite derivative, and (3) removal of the sulphonate group by a subsequent alkali treatment, to give uracil. Bisulphite reacts with cytosine either as the free base, the nucleoside (ribo- or deoxyribo-), the nucleotide or the oligonucleotide. The reaction is highly single-strand specific. Indeed, it can be used to distinguish regions of DNA that are single stranded from those that are double stranded by their differential reactivity (11,12,13).

The first step of the reaction, formation of the sulphonated cytosine derivative (cytosine-SO_3) is reversible (14). Equilibrium is reached much more slowly for DNA than for nucleotides. The extent of adduct formation is controlled by pH, bisulphite concentration and temperature (13). The forward reaction is favoured by low pH and the reverse reaction by high

*To whom correspondence should be addressed at: CSIRO Division of Biomolecular Engineering, Sydney Laboratory, PO Box 184, North Ryde, NSW 2113, Australia

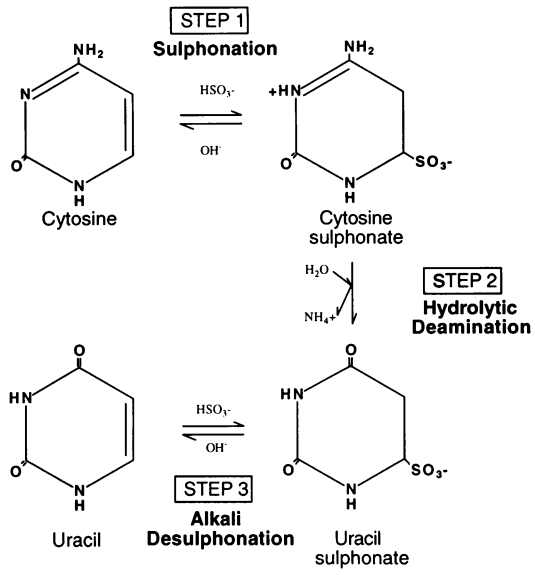

Figure 1. Schematic diagram of the bisulphite conversion reaction.

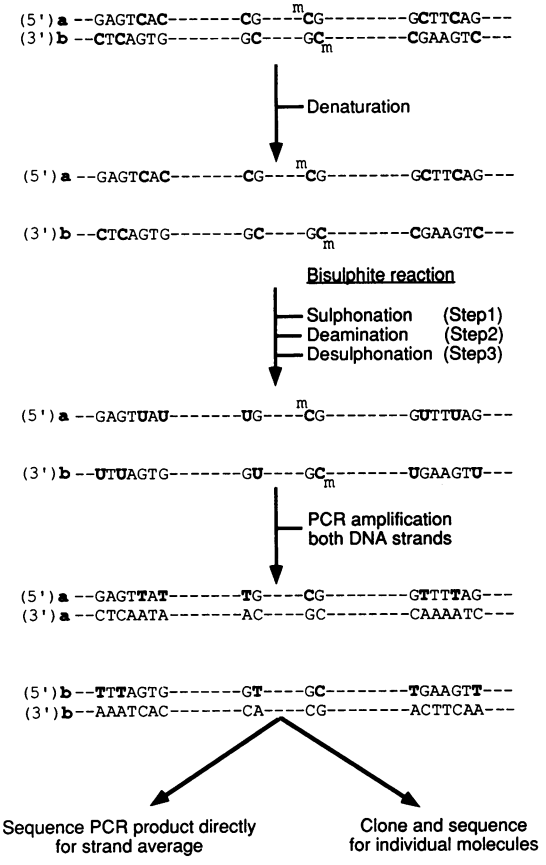

Figure 2. Bisulphite genomic sequencing procedure. The two complementary strands in the original DNA are labelled (**a**) and (**b**). Cytosine residues and their corresponding uracil and thymine conversion products are shown in bold type. It should be noted that, after the bisulphite reaction, the two DNA strands (a) and (b) are no longer complementary and therefore can be amplified independently.

pH. In the second step of the reaction, cytosine-SO_3 undergoes hydrolytic deamination to give uracil-SO_3 (12). This step is catalysed by basic substances, such as sulphite, bisulphite and acetate anions (13). Since sulphonation is favoured by acidic pH, the reversible sulphonation reaction and the subsequent irreversible deamination step are both carried out at a pH below 7. The third step of the reaction involves alkali treatment to remove the bisulphite adduct. Although 5-MeC can also react with bisulphite, the reaction is extremely slow and the equilibrium favours 5-MeC rather than the deaminated product, thymine (11).

Protocols which define the methylation status of every cytosine in a DNA sequence (6,7,10) have required relatively large amounts of DNA (2−100μg). These methods, therefore, have not been readily applicable to the study of methylation in the germline and during early development, when methylation patterns are established and when large scale demethylation and active *de novo* methylation are occurring in a small number of cells. We have now developed the bisulphite genomic sequencing method to analyse the methylation profile of single genes from as little as 200pg of genomic DNA, corresponding to fewer than 100 cells.

MATERIALS AND METHODS

Bisulphite genomic sequencing protocol

1. Bisulphite conversion reaction. The bisulphite reaction was carried out on linear fragments of genomic or plasmid DNA which had been digested with an appropriate restriction enzyme (i.e. one that did not cut within the target sequence). However, if no suitable restriction site is available, for instance when multiple target sequences are to be amplified, DNA can be sheared by passing through a narrow gauge needle. The DNA (200pg−50μg in a volume of 20−200μl) was denatured by adding freshly prepared NaOH to a final concentration of 0.3M and incubating for 15 minutes at 37°C. At this stage, immediately before use, 10mM hydroquinone and 3.6M sodium bisulphite (Sigma) or 2M sodium metabisulphite (BDH), pH 5.0 (pH adjustment with 10M NaOH), were prepared. Degassed water was not used to dissolve the reagents, but solution was achieved by gently inverting the reagent/H_2O mixtures, with minimum mixing and aeration. Sodium bisulphite, to a final concentration of 3.1M, and hydroquinone, to a final concentration of 0.5mM, were added to the denatured DNA to a final volume of 240−2400μl, the sample was gently mixed, overlayed with mineral oil and incubated at 55°C for 16 hours. The DNA was recovered from under the oil layer after snap freezing the reaction and removing the unfrozen oil.

Removal of free bisulphite was achieved by one of two procedures:

(1) The sample was dialysed at 4°C against (a) 2×2 litres 0.5mM hydroquinone/5mM NaOAc pH 5.2 for 1 hour each, (b) 2×2 litres 0.5mM NaOAc pH 5.2 for 1 hour each, (c) 1×2 litres MilliQ H_2O for 1 hour and (d) 1×2 litres MilliQ H_2O overnight. After dialysis he DNA was dried down in a vacuum desiccator until reduced in volume but not completely dried. (2) Alternately, the sample was purified using a desalting column (Promega Magic DNA Clean-Up System), according to the manufacturer's instructions, and eluted in 50μl of H_2O. DNA buffer (10mM Tris, 0.1mM EDTA, pH 8) was added to both dialysed and column-purified samples to bring the volume to 50−200μl.

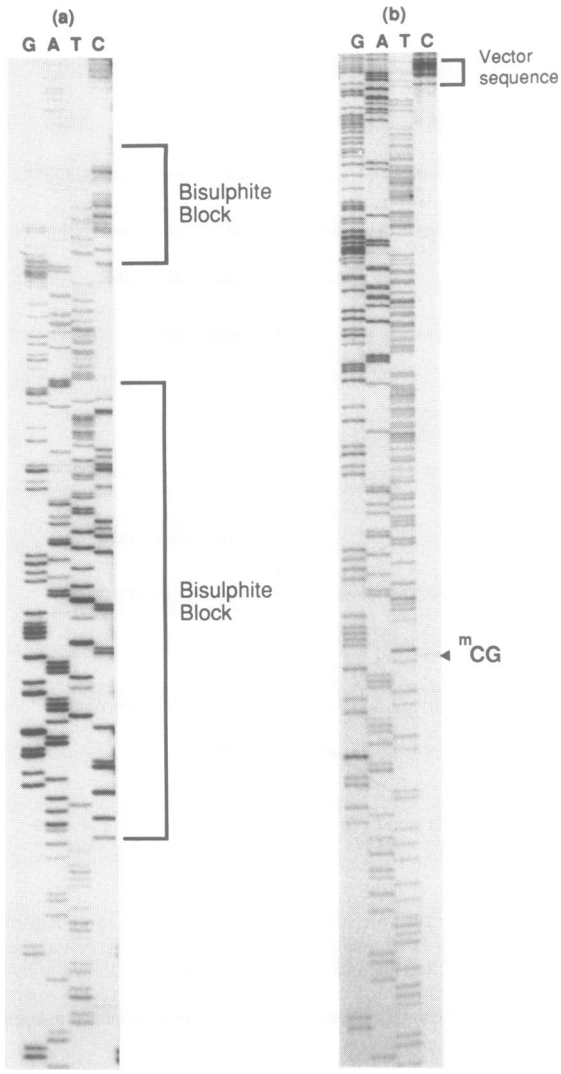

Figure 3. Effect of more stringent denaturation conditions on efficiency of bisulphite conversion. DNA sequence profile of EcoRII methylated pBluescript DNA which was derived from bisulphite-treated mouse genomic DNA spiked with 100 copies of EcoRII methylated plasmid, amplified using specific pBluescript primers designed to bisulphite converted DNA from position 825−854 and 1090−1115 (Stratagene SK sequence). PCR products were cloned into pXT (15) and individual clones sequenced. Prior to bisulphite treatment, the spiked mouse genomic DNA was (a) denatured in 0.2M NaOH and precipitated prior to bisulphite treatment (original conditions), or (b) denatured in 0.3M NaOH, with bisulphite directly added to DNA/NaOH mix.

Freshly prepared NaOH, to a final concentration of 0.3M, was added and the sample incubated at 37°C for 15 minutes. The solution was neutralised by addition of NH_4OAc, pH 7, to 3M and the DNA was ethanol precipitated, dried, resuspended in DNA buffer (100μl) and stored at −20°C.

2. PCR amplification. Amplifications were performed in 100μl reaction mixtures containing 1−5μl of bisulphite-treated genomic DNA, 200μM dNTPs, 1μM primers, 3mM $MgCl_2$, 50mM KC1, 10mM Tris−HCl pH 8.3, 0.5μl (2.5 units) AmpliTaq DNA polymerase (Cetus), in a Corbett Research DNA Thermal Cycler under the following conditions: 94°C/2 min ×1 cycle; 94°C/1 min, 50°C/2 min, 72°C/3 min, ×5 cycles; 94°C/0.5 min, 50°C/2 min, 72°C/1.5 min, ×25 cycles; 72°C/6 min ×1 cycle.

3. Cloning and sequencing. Amplified DNA was ligated into an XcmI T-vector (15) and transformed into competent *E.coli* (DH5α). Cloned DNA was sequenced using Sequenase version 2.0 DNA sequencing kit (USB). PCR products were directly sequenced using the Circumvent Kit (New England Biolabs), with primers end-labelled using T4 polynucleotide kinase and [γ-^{33}P]ATP. To reduce cross banding in some of the sequences, 2.5 units of terminal transferase, in 1×Sequenase reaction buffer, and 1mM dNTPs were added after the cycling reaction, and the mixture was incubated for 30 minutes at 37°C before addition of stop dye.

Optimisation of reaction conditions

Optimisation of each step of the protocol was carried out on mouse L cell genomic DNA spiked with pBluescript (SK) plasmid DNA of known methylation status, at a copy number of 10−100 per genome. Optimised conditions were tested on (1) the same pBluescript sequences, stably integrated at a copy number of 1−10 per genome into F9 and 3T3 cells, and (2) a segment of the mouse endogenous Thy-1 gene. The various bisulphite treatment conditions were assayed in all cases by yield of PCR-amplified products and in some cases by sequencing cloned PCR products or by direct sequencing of PCR products.

RESULTS

Important reaction parameters

We have tested a wide range of parameters at each step of the bisulphite conversion reaction, and have determined which conditions are critical for obtaining a fully-deaminated and amplifiable product. The overall genomic sequencing procedure is outlined in Fig. 2. Among the cloned and sequenced samples, full bisulphite conversion of unmethylated cytosines was observed in most cases. However, the amount of product that could be amplified after bisulphite treatment was highly variable. Of the various parameters tested, we found that there were only a few key factors which determined the extent to which the bisulphite-treated DNA could be readily amplified. These factors were:

(a) Denaturation of the target DNA. The initial denaturation step was found to be critical both for PCR yield and for full deamination. At 0.2M NaOH, the original denaturation conditions, incubation of the DNA followed by ethanol precipitation sometimes resulted in patches of DNA resistant to bisulphite conversion, as shown in Fig.3(a). The pattern of resistance was largely, but not totally, sequence specific, suggesting that the DNA was not being fully denatured prior to incubation with the bisulphite. More stringent denaturation conditions, 0.3M NaOH for 15 minutes at 37°C, resulted in a consistently greater yield of PCR end product. Furthermore, adding the bisulphite directly to the DNA/NaOH mix, instead of firstly precipitating the DNA, also gave a significantly increased yield of PCR product and resolved sequence-specific bisulphite blocks, Fig.3(b).

(b) Bisulphite treatment. The brand of sodium bisulphite or metabisulphite, or the ratio of bisulphite to metabisulphite, used to modify the DNA did not appear to be as critical as the individual batch or length of storage of the chemical. However,

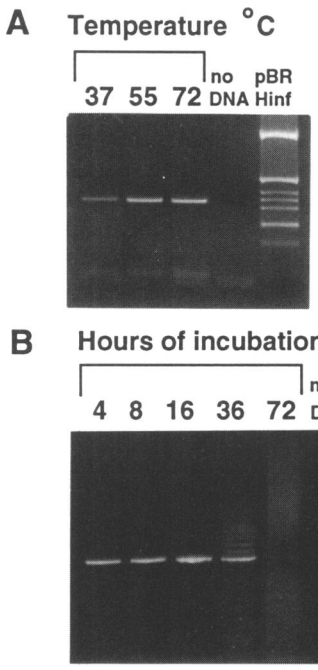

Figure 4. (**A**) Temperature of bisulphite reaction. PCR products derived from mouse L cell genomic DNA (20ng) spiked with 10 copies of Thy1 gene and bisulphite treated, as described in methods, at either 37°C, 55°C or 72°C for 16 hours and amplified using nested Thy1 primers (497−526) and (806−837) designed to converted DNA, as shown in Fig. 8. (**B**) Time course of bisulphite reaction. PCR products derived from mouse L cell genomic DNA (20ng) spiked with 10 copies of Thy1 gene and bisulphite treated, as described in methods, at 55°C for 4, 8, 16, 36, or 72 hours and amplified using nested Thy1 primers (497−526) and (806−837) designed to converted DNA, as shown in Fig.8.

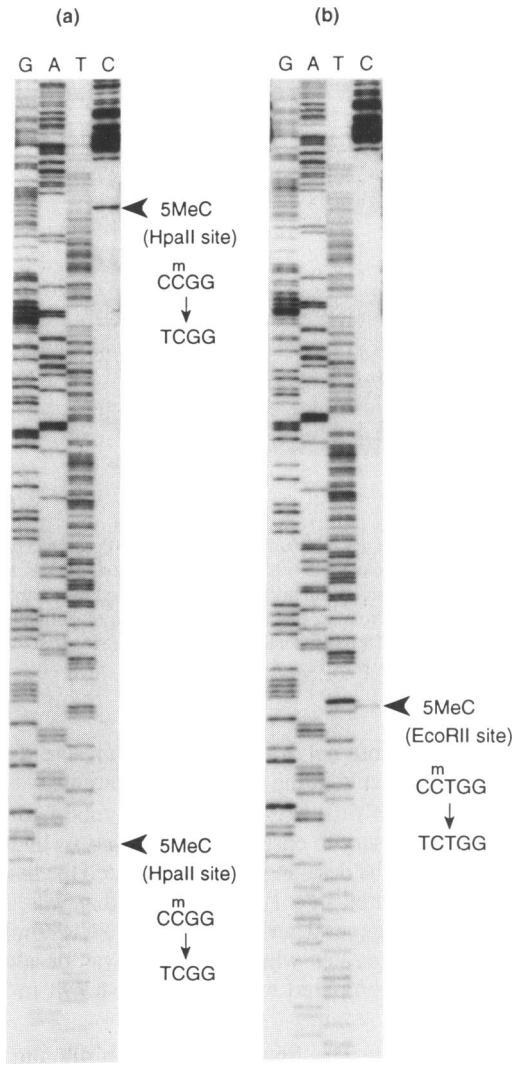

Figure 5. Genomic sequence profile of HpaII and EcoRII methylated pBluescript. PCR products, derived from bisulphite-treated mouse genomic L cell DNA spiked with 100 copies of pBluescript DNA and amplified using specific pBluescript primers, were cloned into pXT (15) and individual clones sequenced. The specific primers were designed to converted DNA, top strand, from position 825−854 (GTGGAATTCTTATTGGTTTATAATTTTATA) and 1090−1115 (AACCGAATTCCCACCTTTAAATAAACTAA) from Stratagene SK sequence. Underlined sequence in primer shows introduced restriction enzyme site and bases in bold indicate thymines corresponding to converted cytosines or adenines complementary to converted cytosines. pBluescript was methylated either at HpaII sites (**a**) or EcoRII sites (**b**).

it is important to ensure that the bisulphite is made fresh immediately prior to use. The molarity of dissolved bisulphite (3.0−4.0M) and pH range (4.8−5.8) of the bisulphite solutions tested had no great effect on PCR amplification. The reaction temperatures (37−72°C) resulted in similar yields of PCR product (Fig. 4A); however this yield decreased when the reaction was performed at higher temperatures. Performing the modification reactions under N_2, using degassed solutions, appeared to be no different to modification reactions incubated under oil.

Time course incubation samples (4, 8, 16, 36, 72 hours) were tested, as shown in Fig. 4B, and of these, the optimal incubation times for DNA with bisulphite were routinely 8−16 hours. Since the rate of depurination of DNA is enhanced by acidic pHs, some random strand breakage may occur when the bisulphite-treated DNA solution is made strongly alkaline to remove the $-SO_3$ adduct from the uracil residues prior to PCR amplification. At longer incubation times, 36 to 72 hours, the PCR products become more diffuse(Fig. 4B) and DNA degradation starts to become evident after the alkali treatment step. This was observed by spiking the bisulphite reactions with labelled HindIII markers and visualising the converted DNA on agarose gels after the final alkali step. However, we have not found degradation to be a major problem under the optimised reaction conditions. In fact, sequencing of time course samples showed that 95% of the cytosines had already been converted to uracils by 8 hours.

The bisulphite reaction conditions tested above made little difference to the yield of PCR product using a number of sets of primers. Many of the PCR products were cloned and the resulting individual clones sequenced. The sequence of the PCR products also showed that there was little difference between the variety of conditions tested. The extent of cytosine deamination was between 98−100% (Fig. 5).

(c) Desalting. We compared various methods to remove the salt from the reaction—dialysis, Centricon filters and desalting columns. We found the DNA loss from Centricon filters unacceptable. We found dialysis to be consistently reliable, especially with the inclusion of the last overnight dialysis step in water, which was crucial in order to reduce the salt concentration. After this step, the DNA was concentrated in the

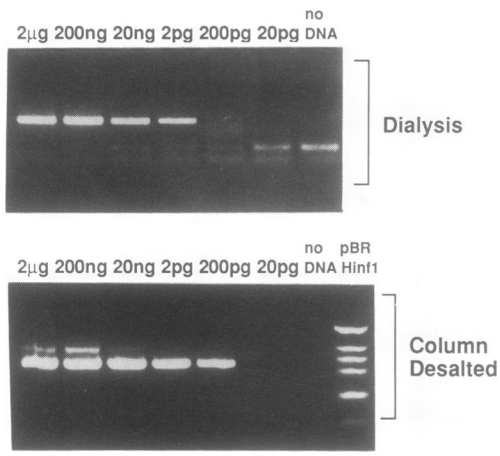

Figure 6. Comparison of desalting using dialysis versus desalting column. PCR products, derived from mouse L cell genomic DNA (2μg−20pg) spiked with 10 copies of Thy1 gene and bisulphite treated as described in methods, desalted either using dialysis or Promega DNA Clean-up System (column desalted) and amplified using nested Thy1 primers (497−526) and (806−837) designed to converted DNA, as shown in Fig. 8.

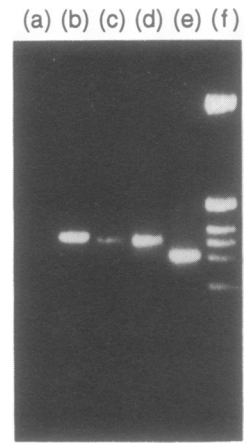

Figure 7. PCR amplifications comparing different primer sets. Mouse genomic DNA was spiked with 100 copies of pBluescript DNA, bisulphite treated as described in methods and amplified with primer sets at different sequence positions, designed to both top and bottom strands of the bisulphite converted DNA. Lanes (a)−(e) show pBluescript-derived PCR products obtained with five different primer pairs, with positions identified by nucleotide number in the Stratagene SK sequence; (**a**) (b)-strand primers: pBluescript 2131−2159 (CTCCGAATTCATCAACAATAAACCAACCA) and pBluescript 2448−2419 (ATAATACTGCAGTTAATTTATTTTTGATAA); (**b**) (a)-strand primers: pBluescript 272−301bases (AGTATTAACTAGTAATTTTAAAGGGAGTTT) and pBluescript 638−609 (CTATAATAAATCCTATTAGAATTCACTAAC); (**c**) (b)-strand primers: pBluescript 284−313 (AAACTCTAGAAAAAACCCCCTATTTAAAAC) and pBluescript 638−609 (TTATAGTGACTAGTATTATAATTTATTGGT); (**d**) (a)-strand primers: pBluescript 1147−1118 (TCTTTCCTACCTTATCCCCGAATTCTATAA) and pBluescript 791−821 (GTTTGGAGGAATTCTGGTTATAGTTGTTTTT); (**e**) (b)-strand primers: pBluescript 1111−1084 (GTATACTAGTTTTTGAGTGAGTTGATAT) and pBluescript 802−833 (CATAATCATAACTAGTTCCTATATAAAATTA). Lane (**f**) contains size markers, PBR322 digested with HinfI. Underlined sequence in primer shows introduced restriction enzyme site and bases in bold indicate thymines corresponding to converted cytosines or adenines complementary to converted cytosines.

vacuum desiccator, but not dried to completion as re-dissolution was sometimes difficult. If the concentrated sample was yellow, we found that it was difficult to amplify the modified DNA. It was therefore necessary to ensure adequate dialysis and frequent changes of buffer. The desalting column provided the most simple procedure and resulted in a PCR substrate that was amplified most efficiently. In general, the sensitivity of PCR amplification was 10−100 fold greater when the DNA was desalted using clean-up columns compared to dialysis, as shown in Fig. 6.

(d) Alkali treatment. The final step of the bisulphite reaction involves removal of the bisulphite adduct from the uracil ring by alkali treatment. It was important that this desulphonation step was complete, and we found that incubating the DNA with 0.3M NaOH for 15 min at 37°C resulted in a consistently greater yield of PCR products than detailed in the original protocol.

(e) Design of primers. Perhaps the most critical step in the bisulphite genomic sequencing protocol is the design of primers to the deaminated DNA. This is the step that amplifies the complementary DNA strands and therefore allows analysis of the methylation status of each individual CpG on both strands of DNA. The primers are designed to favour the amplification of fully bisulphite-converted DNA from a mixture which may also contain some partially converted molecules.

The primers that we successfully used had the following properties:

(i) They were approximately 30bp in length.
(ii) They were designed to an evenly C-rich DNA region.
(iii) They contained a minimum number of CpG dinucleotides, unless the methylation status of CpG residues within the primer region was known. If a CpG dinucleotide within the primer sequence could not be avoided, a mismatch to both the methylated and unmethylated sequence was incorporated into the primer at the C residue of the CpG dinucleotide.
(iv) They showed limited internal complementarity and limited complementary sequences between primer pairs.
(v) They had a minimal number of mismatches to the bisulphite-converted sequence from which the primer was designed, including any mismatches incorporated at CpG dinucleotides and restriction sites.

We found that following these design principles resulted in primers that reliably yielded a PCR product. However, the efficiency of different primers was still quite variable, ranging from very poor (Fig. 7, lane a) to highly efficient (Fig. 7, lane e). It is therefore important to design a number of primers to any target sequence. Furthermore, when amplifying small amounts of DNA, it is desirable to allow for a nested PCR strategy. An example of nested primer design is shown in Fig. 8.

(f) PCR amplification of DNA. Amplifications were optimised for a number of different primers and were consistently found to be improved at 3mM $MgCl_2$ and at 50°C annealing temperature, as described in Materials and Methods. It is important to emphasise that, although these conditions were reliable for our sequences, it may be necessary to optimise PCR conditions for each DNA sequence of interest.

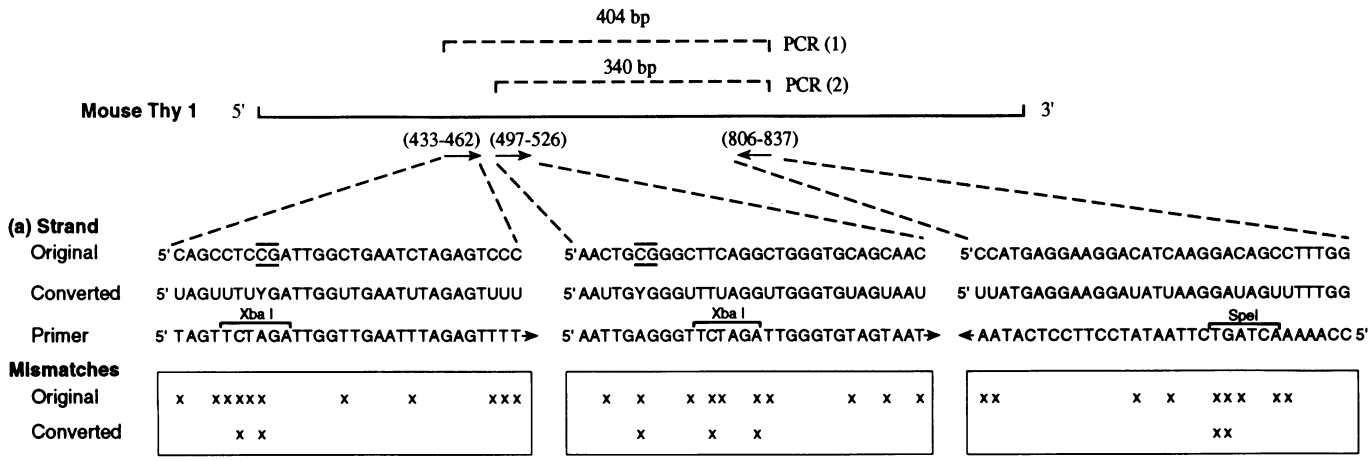

Figure 8. Example of semi-nested primer set, designed for amplification of a segment of the Thy-1 gene from bisulphite-treated mouse genomic DNA. The DNA sequences used to design only the top (a) strand primers are shown. Numbers in brackets give the nucleotide positions of each primer. Original: DNA sequence before bisulphite treatment; Converted: DNA sequence after bisulphite treatment. CpG dinucleotides, methylation status unknown, are marked with lines; restriction sites incorporated into the primers are marked with brackets. U: uracil, Y: thymine or cytosine, x : sequence mismatch. Thy-1 DNA sequences used were those of the mouse Thy-1 1.2 glycoprotein gene (Accession Number M12379, GenBank).

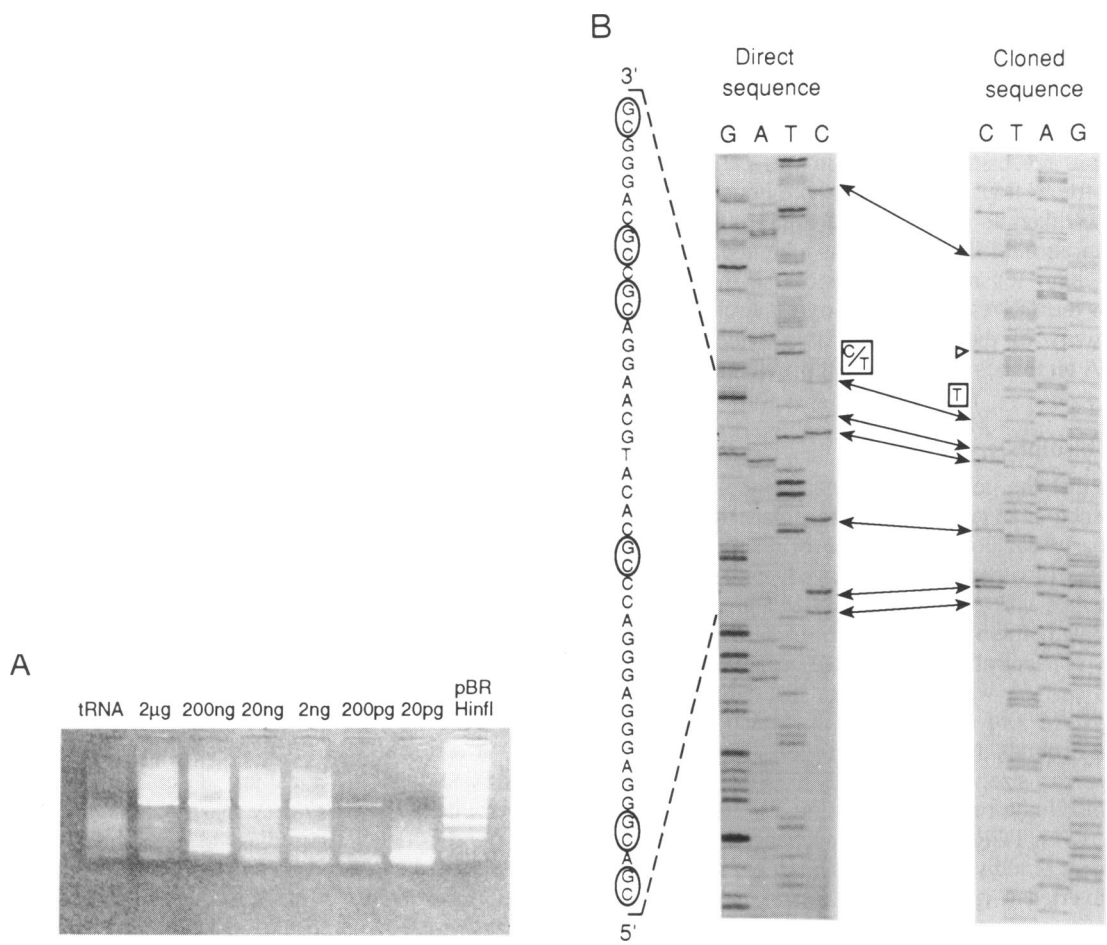

Figure 9. Sensitivity of bisulphite conversion (**A**) PCR amplifications from decreasing amounts of bisulphite-treated mouse genomic DNA (2µg−20pg). The amplifications used the nested primers for Thy-1 converted DNA as detailed in Fig. 8. (**B**) Genomic sequencing gels of the Thy-1 PCR product from the 200pg bisulphite-treated mouse genomic DNA sample. The PCR product was both directly sequenced and cloned into pXT, as shown. Arrows indicate the positions of equivalent CpG dinucleotides. In most cases, the CpG dinucleotides were found to be methylated, as the C residues in these positions remained unconverted by bisulphite. In the direct sequence, one CpG dinucleotide was found to be partially methylated, as indicated by C/T, and unmethylated in the illustrated cloned sequence, as indicated by T. The open arrow indicates the position of a single unconverted cytosine, not in a CpG dinucleotide.

(g) Cloning and sequencing. We routinely cloned amplified DNA and sequenced individual clones. Alternatively, it is possible to sequence the PCR product directly. Direct sequencing allows the overall methylation status of individual cytosine residues to be analysed, whereas sequencing cloned DNA allows the analysis of cytosine methylation in individual molecules.

For almost all sequences, under most conditions, no unreacted cytosine residues were observed. However, we found two regions of DNA in different PCR products where there were patches of up to 100bp of unreacted cytosines in an otherwise fully-converted DNA fragment. In both cases, these unreacted blocks of DNA were only seen when the PCR fragment had been cloned into a pBluescript-based vector and were not present in any clones of the same PCR fragment cloned into a pGem-based vector. It appears that cloning into different vectors may provide some selective advantage for molecules with patches of non-converted sequences over fully-converted molecules. The partially-converted products became evident only when cloning efficiencies were low. When cloning efficiencies were high, pBluescript vector also gave fully-converted products only. We have not observed any blocks of unreacted sequence that were not resolved by changing the vector and increasing the efficiency of the ligation and transformation. However, these products were clearly present as minor components of the PCR amplification. It is possible that some regions of DNA may be intrinsically less reactive with bisulphite because of regions of self complementarity or a particular base sequence. It is interesting to note that Tasheva and Roufa (16) used the protocol of Frommer *et al.* (10) with one modification: the bisulphite reaction was carried out in a thermal cycler for 48 hours, and every three hours the reaction temperature was taken up to 95°C for 5 minutes to ensure that complete denaturation of the sample was maintained. Although this treatment may, to some extent, increase the amount of depurination in the DNA sample, and may therefore limit the absolute sensitivity of the procedure, it provides a possible solution if DNA templates which present problems of reactivity are found.

Sensitivity of bisulphite conversion

It is important that the genomic sequencing method be highly sensitive, to probe changes in methylation patterns of specific genes during early development. In the original protocol, the minimum quantity of genomic DNA assayed was 2μg. Using the protocol described in Materials and Methods, we have successfully scaled down the reaction 10^4 fold. A range of genomic DNA concentrations (2pg−2μg) spiked with 2μg of yeast tRNA as carrier, was treated with bisulphite in a 240μl reaction volume. A 300bp endogenous Thy-1 gene fragment was amplified in two rounds using nested primers (Fig. 8). A PCR product of the correct size was successfully amplified from a 10pg aliquot of the bisulphite reaction carried out on 200pg genomic DNA (Fig. 9A). This PCR product was sequenced, both directly and from a number of individual cloned molecules, and was found to be fully converted (Fig. 9B).

DISCUSSION

Genomic sequencing protocols allow the methylation status of every cytosine residue in genomic DNA to be determined. This information is particularly relevant to studies of methylation in developmental processes and in cancer where time-dependent changes in methylation patterns occur. However, the potential utility of genomic sequencing for methylation has not been realised because the current methods available (6,7) have not been sufficiently sensitive. We have developed the bisulphite genomic sequencing method to determine exact methylation patterns of specific genes in very small amounts of genomic DNA, and the alterations to the method substantially enhance its efficiency and reproducibility. The bisulphite conversion conditions are now sensitive enough to allow methylation analyses of at least 20 gene sequences in DNA isolated from less than 100 cells.

The high sensitivity of the bisulphite method is possible because, during the sequencing procedures, DNA strands remain intact. The bisulphite-treated and amplified DNA is sequenced by the dideoxy protocol, where each DNA strand contributes to every band on a sequencing gel. This means that the theoretical limit of sensitivity of the bisulphite method is one cell, a sensitivity that is not possible in Maxam and Gilbert-derived genomic sequencing protocols, where each strand can only contribute to one band on a sequencing gel.

The non-destructive nature of the bisulphite conversion reaction also means that the PCR product can be cloned and sequenced to provide methylation maps of single DNA strands within a large population of molecules. If partial methylation of sites is observed in a population of DNA molecules, bisulphite genomic sequencing of cloned PCR products gives information on whether correlations exist between the methylation of particular sites on the same DNA molecule, for example, during spreading of methylation from an initiation point. The sequencing of single strands of genomic DNA permits the protocol to be used to identify different methylation patterns in mixed populations of cells or in DNA strands of different parental origin. The protocol has particular utility when local DNA polymorphisms, that do not involve CpG sites, can be identified in the sequence prior to bisulphite treatment.

Important insights into methylation profiles can be gleaned by comparing methylation maps of individual molecules with the methylation map from the population average. Therefore, it is beneficial that PCR products can be sequenced directly to provide a strand-specific average for the population of molecules. Partial methylation at any site can be readily observed in the direct sequence of a PCR product by the presence of both a cytosine and a thymine residue at the same sequencing position (Fig. 9B). Methods for quantitation of the proportion of cytosine and thymine residues at these individual sites in the direct PCR sequence are now being developed using an automated DNA sequencer (C.L. Paul and S.J. Clark, unpublished).

Finally, the bisulphite method provides a positive test for methylation. The position of each 5-MeC residue is indicated by a distinct band on a sequencing gel, in a lane where all cytosine-derived bands are absent, making the location of methylated sites very easy to score. The method now paves the way for detailed studies of the role of methylation in developmental processes such as genomic imprinting, sex determination and X inactivation, as well as in inherited diseases and cancer.

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